

## Pathological Lesions in Chicken Embryo Caused by Newly Virulent Isolate of Newcastle Disease Virus

(LESI PATOLOGIS PADA EMBRIO AYAM YANG DISEBABKAN  
ISOLAT VIRUS PENYAKIT TETELO TERBARU YANG VIRULEN)

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### ABSTRACT

Newcastle disease is a pathogenic viral disease in poultry which is infectious and can cause massive economic losses. The disease is still endemic in Indonesia. To understand the pathogenesis and the distribution pattern of the virus in the tissues, pathological observation was evaluated using newly virulent isolate Newcastle disease virus (NDV) that was inoculated in embryonated chicken eggs. As many as seven embryonic chicken eggs aged 11 days and specific antibody negative against Newcastle disease, divided into two categories: inoculated with phosphate buffer saline and inoculated with isolates. Then the allantois fluid was tested using hemagglutination assay and hemagglutination inhibition tests to prove the infection serologically. The hearts, lungs, livers and small intestines of the inoculated products were collected and followed with the process of histopathological preparation using Hematoxylin and Eosin (HE) stain. The pathological analysis showed that all organs had necrosis, hemorrhages, inflammation, and congestion. Congestion and hemorrhages in the hearts only occurred at 60% of the samples. However, necrosis, hemorrhages, and inflammation that were observed in liver occurred at 60%, 40% and 60% of the samples, respectively. Furthermore, the hearts were edema, thinner in the heart muscle fibers; while in the lungs, proliferation of pneumocyte type II was founded. Our finding provided valuable insight into the pathology of a virulent isolate of NDV which is dominated by blood circulation disorders with necrosis and inflammation in the chicken's embryos and have important implication for the future studies.

Keywords: pathology, chicken embryo, virulent isolate, Newcastle disease virus

### ABSTRAK

Penyakit tetelo atau *Newcastle disease* adalah penyakit virus patogenik pada unggas yang bersifat infeksius dan dapat menyebabkan kerugian ekonomi yang signifikan. Penyakit tetelo tersebut masih endemik di Indonesia. Untuk memahami patogenesis dan pola distribusi penyakit pada jaringan, pengamatan lesi patologis dilakukan dengan menggunakan virus *Newcastle disease* isolat virulen terbaru yang diinokulasi pada telur embrio bertunas. Tujuh telur ayam berembrio berumur 11 hari yang tidak memiliki antibodi spesifik terhadap virus tetelo atau *Newcastle disease virus* (NDV), dibagi menjadi dua kategori yakni: diinokulasi dengan *phosphate buffer saline* dan diinokulasi dengan isolat. Kemudian cairan alantois diuji dengan uji hemaglutinasi dan hambatan hemaglutinasi untuk membuktikan infeksi secara serologis. Organ jantung, paru-paru, hati dan usus halus hasil inokulasi diambil dan diproses untuk pembuatan preparat histopatologis dengan pewarnaan Hematoksin dan Eosin (HE). Pengamatan lesi patologis menunjukkan, bahwa hampir semua jaringan atau organ mengalami nekrosis, perdarahan, peradangan, dan kongesti. Kongesti dan perdarahan di jantung hanya terjadi pada 60% dari semua sampel. Namun, nekrosis, perdarahan, dan peradangan pada hati yang terjadi sebanyak 60%, 40% dan 60% dari keseluruhan

sampel secara berturut-turut. Selain itu, jantung tampak edema, penipisan serabut otot jantung, sedangkan pada paru-paru terjadi proliferasi pneumosit tipe II. Temuan ini memberikan tambahan pengetahuan bahwa lesi patologis isolat NDV ganas didominasi oleh gangguan sirkulasi darah pada embrio ayam dan memiliki implikasi penting untuk studi masa depan.

Kata-kata kunci: embrio ayam; isolat virulen; virus tetelo; Newcastle disease virus

## INTRODUCTION

Newcastle disease (ND) is a viral disease with a high mortality rate that affecting many of avian species. This virus is reported to be endemic and causes epizootic outbreak in domestic poultry (Miller *et al.*, 2010). In South-East Asia, the disease is also endemic, including in Indonesia (Adi *et al.*, 2010). The outbreak of the disease still commonly occurs in poultry and causes massive economic losses, although vaccination and biosecurity have been conducted intensively (Antipas *et al.*, 2012; Qin *et al.*, 2008).

Newcastle disease caused by virulent strains of ND virus (NDV). The virus possesses a non-segmented negative-sense single-stranded RNA. The RNA contains six genes, i.e.: nucleocapsid protein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase protein (HN), and large polymerase protein (L) in which HN and F proteins play important roles during the infections to the host cells (OIE, 2012; Yan and Samal, 2008; Miller *et al.*, 2010; Fournier and Schirrmacher, 2013; Swanson, 2010). Today, NDV is still evolving into different genotype which may lead to diagnostic failures and affect more severe economy (Miller *et al.*, 2010; Perozo *et al.*, 2008; Rue *et al.*, 2010).

Newcastle disease virus does not only infect adult chickens, but it also infects the embryo (Swayne *et al.*, 2013). Bwala *et al.* (2012), reported by using immunohistochemistry stain, some NDVs have a tropism in the oviduct that possibility lead to the virus transmit directly into the embryo.

Despite the ND is one of notable disease in the poultry, there is only a few studies on the pathology of NDV that infected the chicken embryo, especially from field isolate case in Indonesia. This research aimed to determine pathological observation and the lesion distribution pattern in internal organs of the chicken embryo due to inoculation of the newly isolate Tabanan-1/ARP/2017.

## RESEARCH METHODS

### Embryonic Chicken Eggs and Virus

Seven embryonic chicken eggs (ECE) from white leghorn chicken aged eleven days, were used in this study. The ECEs were obtained from Animal Disease Investigation Center (DIC) Denpasar, Bali. ECEs comes from a parent who has never been vaccinated which is kept specifically for the purpose of testing and research only and has been proved to be specific antibody negative (SAN) against NDV.

The virulent isolate of NDV Tabanan 1/ARP/2017 (Adi *et al.*, 2019) that belongs to genotype VII was used to inoculate the ECEs. This isolate was obtained from NDV infected chicken in a vaccinated laying farm. The isolate was grouped into virulent virus as it has been sequenced and the genome of the virus was found that the amino acid motif at the fusion (F) enzyme cleavage site was <sup>112</sup>R-R-Q-K-R-F<sup>117</sup>.

### Virus Inoculation

Seven ECEs were randomly distributed into two experimental groups, consisted of five embryos that inoculated with NDV Tabanan 1/ARP/2017 as the infected group; whereas two embryos were used as a control group which inoculated only with phosphate-buffered saline (PBS). The infected embryos were inoculated with 0.2 mL suspension containing of 2<sup>7</sup> Hemagglutinin Unit (HAU) virus via allantois route; while the control group treated with 0.2 mL PBS each with the same route. The ECEs then incubated and observed every 12 hours until the embryos seen dead. After that, the allantois fluid was collected and tested by using Hemagglutinin assay (HA) and Hemagglutinin inhibition (HI) test to confirm NDV serologically.

### Haemagglutination Assay (HA) and Haemagglutination inhibition (HI) Test

This HA and HI test used was based on the standard of Office International des Epizootie (OIE) with minor modification (OIE, 2012). As much as 0.025 mL of PBS was dispensed into

each well of a microtitre plate. Then 0.025 mL of the virus suspension was placed in the first well and diluted as much as two fold. A further 0.025 mL of PBS and 1% chicken RBCs were added, mixed and incubated for about 40 minutes at room temperature. Afterward, the result was read and calculated.

In the HI test, the 0.025 mL of PBS was dispensed into each well of the plate. As much as 0.025 mL of serum was placed into the first well and diluted as much as two fold. Four HAU virus/antigen in 0.025 mL was added to each well and incubated for 30 minutes at room temperature. Then 0.025 mL of 1% chicken RBCs was added to each well, mixed and incubated for about 40 minutes at room temperature then the result can be seen. The HI test using avian influenza (AI) antibody was also tested to confirm coinfection with NDV.

### Histopathological Examination

Tissue samples of the embryos were collected from the lung, heart, liver, and small intestine. The collected organs then fixed in 10% neutral buffered formalin, dehydrated using distilled alcohol, clearing, embedded with paraffin, blocked, sectioned, and finally stained by hematoxylin and eosin (HE). All tissues were observed under a light microscope, and the lesions were recorded.

## RESULTS AND DISCUSSION

Based on the HA test result, the titre from the infected group was relatively high with two ECE at  $2^9$  and three ECE at  $2^8$  (Table 1). This means that the HA test is positive containing the antigen that have hemagglutinin and consequently the titre is higher compared to the standart ( $2^2$ ). The difference result of the titer can be influenced by the virulence of the virus. The higher the titer, the more virulent the virus (AbsalAn *et al.*, 2012). This result shows the maximum of the HA test result in this research was  $2^9$ , therefore, the isolate of Tabanan 1/ARP/2017 indicates its virulent. Then, based on this result, the test could continue to the HI test (OIE, 2012).

The results of HI test to NDV were two ECEs titer at  $2^8$  and three ECEs titer at  $2^7$  (Table 1). According to OIE (2012), the result of the HI test was positive as the minimum titer to get a positive result was  $2^4$ . The result means that the mortality of our sample was caused by NDV serologically. To confirm that there is no avian

influenza (AI) virus infection, the HI test for AI was also conducted. The result of the test showed that it means there is no coinfection with AI that occurred in the embryos (data not shown).

Histopathological examination in all organs of the infected group revealed necrosis, hemorrhage, inflammation, and congestion. Furthermore, heart edema, thinner of heart muscle fiber and proliferation of pneumocytes type II also founded in the lung (Figure 1). Congestion and hemorrhage in heart only occurred in 60% samples. Moreover, necrosis, hemorrhage, and inflammation in liver also found in 60%, 40% and 60% samples in this research respectively. The other lesions were found in all (100%) of the sample (Table 2). On the other hand, there was no lesion found in the control group.

The similar finding was reported when using Long Bien isolate (genotype VIIId) that shown severe necrosis of the epithelium with a deposit of the cell debris within the intestinal lumen in birds (Susta *et al.*, 2011). The other similar finding was described by Zhang *et al.* (2011) in China by using SD-09 isolate (genotype VII) which result in the disappearing of the epithelium and intense inflammation in small intestine. The congestion with hemorrhage also was observed in the lung of the chicken. The similar result from the previous research with this study indicates that the virus with the same genotype (genotype VII) can cause the similar result pathologically even though not using the same host.

However, a different result was reported when using Salatiga isolate inoculated in the chicken embryo with congestion and hemorrhage in lungs together with congestion in intestines, liver, and heart (Putra *et al.*, 2012). Other different results also found when the virus isolate inoculated into adult chickens, for example, a study in West Java discover pneumonia, pericarditis, myocarditis, catarrhal enteritis, and hepatitis (Etriwati *et al.*, 2017) and in India using Genotype XIII of NDV shown necrosis, degeneration, hemorrhages, and mononuclear cell diffuse infiltration in visceral organs of adult chicken (Khorajiya *et al.*, 2015). Susta *et al.* (2015) prove using NDV genotype XIV (Niger/06 isolate) and genotype XVII (isolate of Nigeria/06 and BF/08) that obtain from West Africa which inoculate into adult chicken showing necrosis, dulling of villous, mucosal erosion, segmented hemorrhagic and the depletion of lymphoid in intestine together with minimal lesion in

Table 1. Characteristic of HA and HI titer of sample allantoic's fluid and embryo condition post inoculation of NDV isolate

Group	Character of infected allantois' fluid		The presence of embryonic death
	HA Test (HA titre)	HI Test (HI titre)	
Control a	Negative	Negative	Negative
Control b	Negative	Negative	Negative
Infected a	2 <sup>9</sup>	2 <sup>8</sup>	Positive
Infected b	2 <sup>9</sup>	2 <sup>8</sup>	Positive
Infected c	2 <sup>8</sup>	2 <sup>7</sup>	Positive
Infected d	2 <sup>8</sup>	2 <sup>7</sup>	Positive
Infected e	2 <sup>8</sup>	2 <sup>7</sup>	Positive

Note: HA= hemagglutination; HI= hemagglutination inhibition

Table 2. Prominent lesion of chicken embryos tissues after infected by NDV isolate

Organ	Lesion	Group	
		Control (a/b) *	Infected (a/b) *
Heart	Necrosis	0/2	5/5
	Hemorrhage	0/2	2/5
	Inflammation	0/2	5/5
	Congestion	0/2	2/5
	Edema	0/2	5/5
	The thinner of heart muscle fiber	0/2	5/5
Lung	Necrosis	0/2	5/5
	Hemorrhage	0/2	5/5
	Inflammation	0/2	5/5
	congestion	0/2	5/5
	The proliferation of pneumocyte type II	0/2	5/5
Liver	Necrosis	0/2	3/5
	Hemorrhage	0/2	2/5
	Inflammation	0/2	3/5
	Congestion	0/2	5/5
Small intestine	Necrosis	0/2	5/5
	HHemorrhage	0/2	5/5
	Inflammation	0/2	5/5

respiratory organ. Therefore, it seems that the origin and distinct genotype of the isolate may affect the differential of pathology changes of the infected chicken or its embryos.

Extensive infiltration of inflammatory cells and necrosis of lymphoid tissues especially in the intestine that we obtained from this research prove that the Tabanan isolates pathologically belong into typical virulent strain which has more severe lesion than non-virulent strain (Courtney *et al.*, 2013; Diel *et al.*, 2012). The virulent NDV molecularly has amino acid sequenced at fusion (F) enzyme cleavage site is <sup>112</sup>R/K-R-Q/K/R-K/R-R-F<sup>117</sup>. This sequence

capable to cleave easily intracellular by ubiquitous furin-like proteases that found in most host tissues that can cause systemic and often fatal infection with high mortality (Farooq *et al.*, 2012; OIE, 2012; Vidanovic *et al.*, 2012). However, the non-virulent NDV that cleaved extracellularly by a trypsin-like protease which generally only takes place in intestinal and respiratory tracts (Merino *et al.*, 2011).

The heavily infected of intestinal from this research result prove that the virulent isolates NDV that used in this study is viscerotropic, similar with the previous study using isolate from Indonesian (Adi *et al.*, 2010). In contrast,

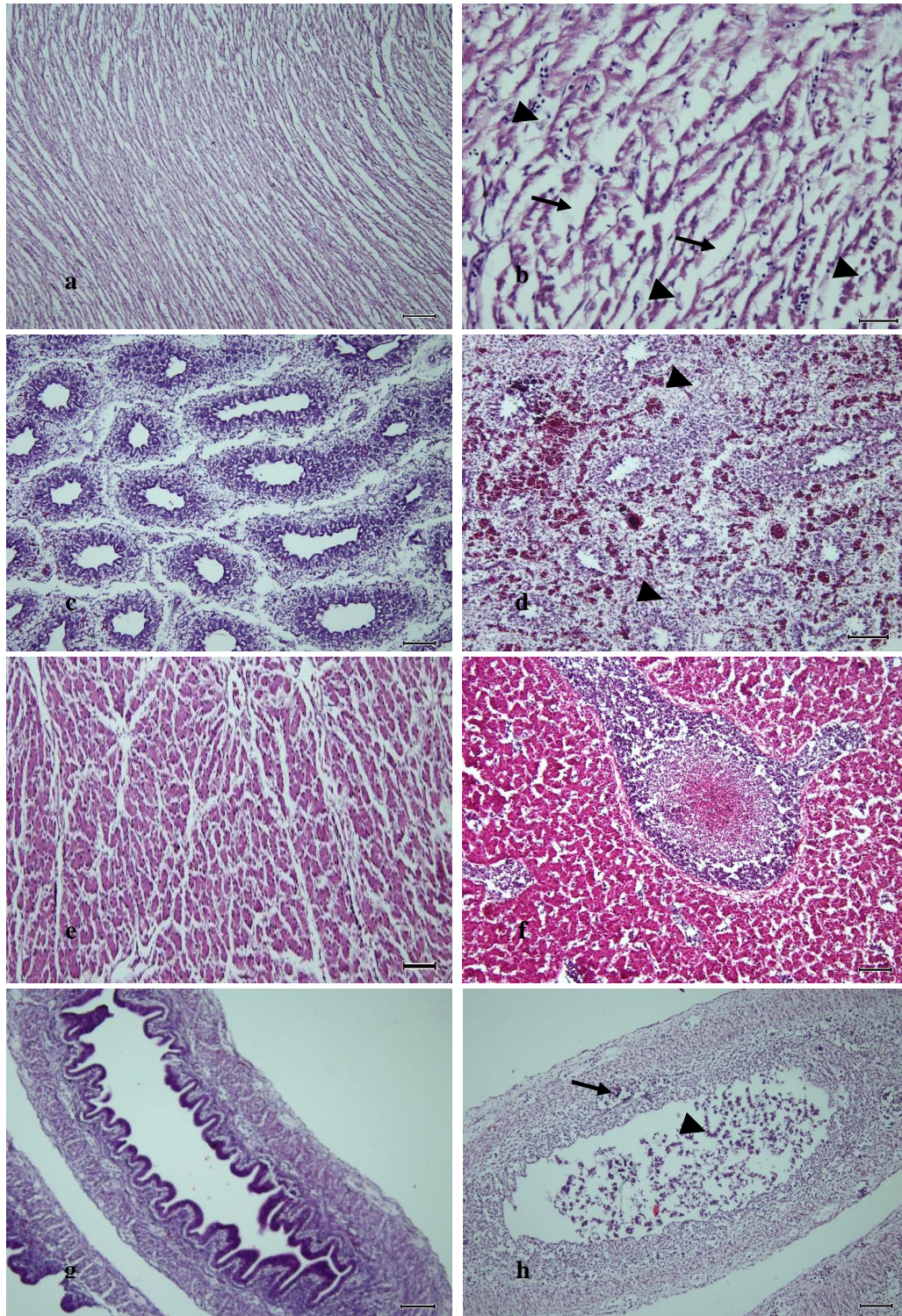


Figure 1. Histopathology of the organ that collected from the chicken embryo. **a** Heart from the control group (Bar 100  $\mu$ m). **b** Edema (arrow), necrosis myocardium (arrowhead) and mononuclear cells infiltration of heart can be seen in infected group (Bar 30  $\mu$ m). **c** Atelectasis of lung in control group occurs because the embryo has not breathed (Bar 100  $\mu$ m). **d** Necrosis (arrowhead), the proliferation of pneumocyte type II, mononuclear cells infiltration, diffuse hemorrhage and congestion founded in lung infected group (Bar 30  $\mu$ m). **e** Liver control group (Bar 30  $\mu$ m). **f** Congestion and mononuclear cells infiltration can be observed in liver from the infected group (Bar 30  $\mu$ m). **g** Small intestine from the control group. **h** Small intestine of the infected group can be seen debris cell from necrotic villi of the intestine (arrowhead), congestion (arrow) and diffused mononuclear cells infiltration (Bar 30  $\mu$ m). HE stains

the NDV that commonly found in North America and Europe is neurotropic type with neural clinical sign like torticollis (Ecco *et al.*, 2011). The neurotropic NDV mainly infecting endothelial cells and neuronal cells such as neuron and astrocytes (Nakamura *et al.*, 2008; Susta *et al.*, 2011).

Beside the edema that only occurs in the heart, we found that the circulation disorder like hemorrhage and congestion almost occur in all organs of our samples. The virus replication in the intestinal lymphoid follicles can cause ulceration and circulation disorders in the internal organ that can lead to viremia (Eze *et al.*, 2014). The viremia also proves that all organs that we observed in the embryo showing pathological lesions. That statement also stated by Etriwati *et al.* (2017) whom state the velogenic type virus inoculate into adult chicken can be found in the vascular endothelial cells by using immunochemistry which means the virus can circulate through the vascular vessel and replicate in the endothelial vascular to create a circulation disorder.

The intensity of lesions in the tissues varies also depending on the quantity of the virus that infect in the organ sampling. In this study, the highest intensity of lesions found in small intestine and lungs. While the lesions intensity in the heart and liver are less severe when compared to the other two organs. Although virulent NDV is able to infect and multiply in almost all of the cells in the chicken (Kattenbelt *et al.*, 2006; Wakamatsu *et al.*, 2006) in this study, the intensity of the lesions found is not same in all organ samples. There are several possibilities that affect the results of this research among others like the number of viruses present in the organ is still relatively low at the time of death so the lesion appears to be slight or because of the NDV tends to like certain types of cells (tropism) so that lesions tend to be more severe in the several organs. This is described in the study of Adi *et al.* (2012) which states that high-intensity of positive antigen presenting cell against ND virus is always found in lymphoid organs, lungs and intestines so that organs tend to have more and severe lesions than others.

The isolate that used in this research we obtain from vaccinated layer farm showed that the preventing the disease using vaccine in the field is not really successful. At present, ND vaccination cannot prevent NDV replication that proves by virus that still found in feces after

vaccination, especially the virulent NDV (Miller *et al.*, 2009; Kapczynski *et al.*, 2013). The failure in vaccination program can be affected by various factors, i.e.: virus factor, human factor and environmental factor. The other study also described the ability of NDV to past the chicken physical protection and antibody with high speed replication in the cell may also the causes of vaccination failure (Kapczynski *et al.*, 2013; Wang *et al.*, 2015).

## CONCLUSION

The newly isolated Tabanan-1/ARP/2017 in chicken embryo can cause lesions which is dominated by blood circulation disorder with necrosis and inflammation that was in hearts, lungs, livers and small intestines.

## SUGGESTION

In the present study, the isolate was an isolate circulating in laying chicken farms that had been vaccinated. Therefore, it needs to be explored whether this isolate can be neutralized by antibodies from vaccination with commercial vaccines or not.

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